**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 5 : | | (11) International Publication Number: WO 94/00464 |
|---|---|---|
| C07H 1/08, C12N 9/99<br>C12Q 1/68, C07K 3/24 | A1 | (43) International Publication Date: 6 January 1994 (06.01.94) |

(21) International Application Number: PCT/US93/04576

(22) International Filing Date: 13 May 1993 (13.05.93)

(30) Priority data:
07/902,978 23 June 1992 (23.06.92) US

(71) Applicant: MINNESOTA MINING AND MANUFACTURING COMPANY [US/US]; 3M Center, Post Office Box 33427, Saint Paul, MN 55133-3427 (US).

(72) Inventors: BITNER, Rex, M. ; CHAN-WHA, Kim ; WILLIAMS, Michael, G. ; Post Office Box 33427, Saint Paul, MN 55133-3427 (US).

(74) Agents: HORNICKEL, John, H. et al.; Minnesota Mining and Manufacturing Company, Office of Intellectual Property Counsel, Post Office Box 33427, Saint Paul, MN 55133-3427 (US).

(81) Designated States: CA, JP, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
*With international search report.*

(54) Title: DEPROTEINIZATION WITH AZLACTONE-FUNCTIONAL SUPPORTS

(57) Abstract

A method of using azlactone-functional supports to separate proteinaceous materials from non-proteinaceous materials is disclosed. Deproteinization with azlactone-functional supports can retain biological activity of the non-proteinaceous material and can bind both net-positive and net-negative proteinaceous materials from biological fluids. A method for controlling restriction enzyme activities in a biological fluid is also disclosed.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|---|---|---|---|---|---|
| AT | Austria | FR | France | MR | Mauritania |
| AU | Australia | GA | Gabon | MW | Malawi |
| BB | Barbados | GB | United Kingdom | NE | Niger |
| BE | Belgium | GN | Guinea | NL | Netherlands |
| BF | Burkina Faso | GR | Greece | NO | Norway |
| BG | Bulgaria | HU | Hungary | NZ | New Zealand |
| BJ | Benin | IE | Ireland | PL | Poland |
| BR | Brazil | IT | Italy | PT | Portugal |
| BY | Belarus | JP | Japan | RO | Romania |
| CA | Canada | KP | Democratic People's Republic of Korea | RU | Russian Federation |
| CF | Central African Republic | | | SD | Sudan |
| CG | Congo | KR | Republic of Korea | SE | Sweden |
| CH | Switzerland | KZ | Kazakhstan | SI | Slovenia |
| CI | Côte d'Ivoire | LI | Liechtenstein | SK | Slovak Republic |
| CM | Cameroon | LK | Sri Lanka | SN | Senegal |
| CN | China | LU | Luxembourg | TD | Chad |
| CS | Czechoslovakia | LV | Latvia | TG | Togo |
| CZ | Czech Republic | MC | Monaco | UA | Ukraine |
| DE | Germany | MG | Madagascar | US | United States of America |
| DK | Denmark | ML | Mali | UZ | Uzbekistan |
| ES | Spain | MN | Mongolia | VN | Viet Nam |
| FI | Finland | | | | |

# DEPROTEINIZATION WITH AZLACTONE-FUNCTIONAL SUPPORTS

## FIELD OF THE INVENTION

This invention relates to the use of azlactone-functional supports to separate non-proteinaceous material from proteinaceous material, especially when such materials are in biological fluids.

## BACKGROUND OF THE INVENTION

Deproteinization is often an essential requirement in the production or separation of high quality non-proteinaceous materials. Non-proteinaceous materials include polysaccharides, steroids, alkaloids, lipids, and genetic materials such as nucleic acids, nucleotides, plasmids. However, a major difficulty occurs because of a lack of a sufficient driving force to differentiate non-proteinaceous materials from proteinaceous materials. Proteinaceous materials include amino acids, peptides, proteins, glycoproteins, lipoproteins, and others.

Current deproteinization processes, such as solvent precipitation, acid precipitation, salting-out procedures, ion-exchange adsorption, heat denaturation, ultra-filtration, ultra-centrifugation, and dialysis, are time consuming and costly or ineffective.

U.S. Patent 4,421,653 discloses a process for the deproteinization of biological fluids by removing the water insoluble phase after adding to the fluid a water insoluble cross-linked polycarbonic polymer. A water insoluble cross-linked diamine (ethylenediamine or hexamethylenediamine) polycarbonic acid is one of the deproteinizing agents employed.

However, the polyvalent cationic surface of the diamine polycarbonic acids provides non-specific adsorption of non-proteinaceous materials, such as nucleic acids and polysaccharides, which should be retained in the solution after deproteinization process for further recovery or analysis.

Additionally, U.S. Patent 4,923,978 discloses a process for separating proteinaceous materials from nucleic acids involving contacting a solution containing the proteinaceous materials and nucleic acids with solid phase extraction materials capable of binding proteins to form bound and unbound fractions and then isolating the unbound fraction containing the nucleic acids. Rehydrated silica gel is one of the solid phase extraction materials employed, but the surface of the silica gel must be freed of polyvalent cationic species if nucleic acids are to be prevented from being adsorbed on the surface of the silica

particles. Three methods are disclosed to achieve a solid phase extraction material which is useful. But such methods involve difficult and time consuming processes.

U.S. Patent 5,004,806 (Kung) discloses a method to remove proteins from a solution containing polynucleotides and proteins by filtering the solution through a nitrocellulose membrane at neutral or basic pH. However, this method is confined to filtration techniques which do not completely deproteinize the solution nor provide efficient DNA retention in the filtrate when using neutral pH values.

SUMMARY OF THE INVENTION

The problem of finding a method to separate proteinaceous materials from non-proteinaceous materials in an effective and efficient manner is solved by the use of azlactone-functional supports which selectively interact with azlactone-reactive amine moieties in proteinaceous materials. Amine moieties are a differentiating factor between proteinaceous materials and non-proteinaceous materials.

Azlactone-functional supports selectively separate proteinaceous materials from non-proteinaceous materials. This separation can be made in fluids where both non-proteinaceous materials and proteinaceous materials are found, especially in biological fluids. Nonlimiting examples of biological fluids include whole blood, plasma, sera, lymph, bile, urine, fermentation liquor, spinal fluid, sputum, sweat, microbial culture filtrate, cell lysate, biological extracts, and fluid preparation from biological tissue. Preferably, for the production of high quality non-proteinaceous materials or for the preparation of analytical or diagnostic test samples, deproteinization is achieved by the binding of proteinaceous material to azlactone-functional supports.

A method of deproteinizing materials according to the present invention comprises separating proteinaceous material from non-proteinaceous material by binding proteinaceous material to an azlactone-functional support.

Another method of the invention comprises contacting an azlactone-functional support with a biological fluid containing proteinaceous material and non-proteinaceous material to bind proteinaceous materials to the azlactone-functional support, and separating the azlactone-functional support having proteinaceous material bound thereto from the fluid containing the non-proteinaceous material.

Another method of the invention comprises controlling restriction enzyme activities in a biological fluid comprising non-proteinaceous, genetic material and restriction enzyme. The method

comprises (a) contacting an azlactone-functional support with biological fluid; (b) inactivating the restriction enzyme in the biological fluid by binding the restriction enzyme to the azlactone-functional support; and (c) separating the azlactone-functional support from the biological fluid to remove inactivated restriction enzyme from the biological fluid.

It is a feature of the present invention that non-proteinaceous materials are separated from proteinaceous materials by binding of the proteinaceous materials to azlactone-functional supports.

It is another feature of the present invention that deproteinization of biological fluids is useful in the production or separation of non-proteinaceous materials.

It is another feature of the present invention that deproteinization of biological fluids is useful for the preparation of analytical or diagnostic test samples.

It is another feature of the present invention that azlactone-functional supports can separate positively charged proteinaceous material, negatively charged proteinaceous material, and uncharged proteinaceous material from non-proteinaceous material.

It is an advantage of the present invention that the separation of proteinaceous material from non-proteinaceous material according to the present invention is achieved in an effective and efficient manner.

It is another advantage of the present invention that biologically active non-proteinaceous material separated according to the process of the present invention retains its biological activity and is otherwise unaffected by methods of the present invention.

Embodiments of the invention follow a brief description of the drawing.

## BRIEF DESCRIPTION OF THE DRAWING

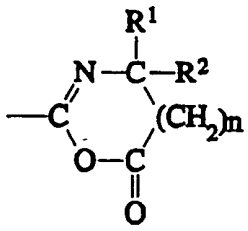
Fig. 1 is a graph showing the adsorption and removal of a proteinaceous material from a solution using azlactone-functional particles.

Fig. 2 is a graph showing adsorption kinetics of two proteinaceous materials on azlactone-functional particles.

## EMBODIMENTS OF THE INVENTION

### Azlactone-Functional Supports

Azlactone-functional supports are materials having units of the formula:



I

wherein $R^1$ and $R^2$ independently can be an alkyl group having 1 to 14 carbon atoms, a cycloalkyl group having 3 to 14 carbon atoms, an aryl group having 5 to 12 ring atoms, an arenyl group having 6 to 26 carbon and 0 to 3 S, N, and nonperoxidic O heteroatoms, or $R^1$ and $R^2$ taken together with the carbon to which they are joined can form a carbocyclic ring containing 4 to 12 ring atoms, and
n is an integer 0 or 1.

Azlactone-functional supports useful in the methods of the present invention can be in the form of an article comprising azlactone-functional polymer or an azlactone-functional polymer coated on at least one surface of a substrate. Nonlimiting examples of azlactone-functional articles include films, webs, membranes, particles (such as beads), articles (such as microtiter wells or test tubes), and other structures from which the azlactone-functional unit of formula I above can be available for interaction with proteinaceous materials.

The structure of various polymeric supports having azlactone functional units of formula I above and their methods of preparation are disclosed in U.S. Patent 4,871,824 (Heilmann, et al.) and European Patent Publication 0 392 735 (Heilmann, et al.). Such azlactone-functional materials can be used in the method of the present invention.

Azlactone graft copolymers and methods of their preparation are disclosed in U.S. Patent 5,013,795 (Coleman, et al.) and European Patent Publication 0 392 783. Such azlactone-functional materials are useful in the method of the present invention.

Azlactone-functional supports are also disclosed in International Application Serial No. US 92/07659 (Rasmussen et al.) and in International Application Serial No. US 93/_____ (Gagnon et al.) (Attorney Docket 45471PCT2A).

When the azlactone-functional support is in the form of particles, the size of the particles can be from about 0.1 to 1,000 μm and preferably 0.5 to 100 μm. The particles can be porous or non-porous. When porous, the porosity of the particles can range from about 1 to about 1,000 Angstroms and preferably from about 10 to about 500 Angstroms.

When in the form of a membrane or web, the porosity of the azlactone-functional supports should be of a size to permit access of proteinaceous materials to surfaces of the membrane or web, and generally can have physical characteristics described in Rasmussen et al. and Gagnon et al. identified above.

The degree of deproteinization according to the present invention depends on the density of azlactone units of formula I above on the surface of the azlactone-functional support, the available surface area of the support having azlactone-functionality, and when the support is in the form of a particle, the size of the particle.

DEPROTEINIZATION METHODS

Azlactone-functional supports can be used to separate proteinaceous materials from non-proteinaceous materials in several manners.

When azlactone-functional supports are in the form of particles, they can be added to a vessel containing proteinaceous and non-proteinaceous materials in a fluid. After gentle agitation between about 1 to 10 minutes, such particles can be removed by centrifugation, skimming, filtration or sedimentation. Removal of azlactone-containing particles from the vessel leaves a deproteinized supernatant containing non-proteinaceous materials for further processing.

Azlactone-functional supports in the form of particles can also be packed into columns through which fluids, especially biological fluids, can be drawn using a variety of chromatographic techniques. Non-limiting examples of such chromatographic techniques include high pressure liquid chromatography, spin column chromatography, mini column chromatography, and the like.

Azlactone-functional supports in the form of membranes or webs can be used in the method of the present invention in microtiter wells, filters, dialysis tubing, linings on walls of vessels containing such proteinaceous materials or transport structures having such proteinaceous materials passing through, and the like.

When the azlactone-functional supports are in the form of articles such as microtiter wells or test tubes, proteinaceous

material is retained in the article and the deproteinized supernatant is removed for further processing.

When it is desired to remove proteinaceous material from the biological fluids described above, the degree of deproteinization can vary according to the number of azlactone-functional groups that are available and the amount of proteinaceous material in the biological fluid. The temperature, pH-value, ionic strength, and buffering condition are, in principle, not critical. However, the temperature generally lies between 0 to 100°C, preferably between 4°C and 40°C, and the pH-value between 0 and 11, desirably between 4 and 11, and preferably between 6 and 10. When deproteinizing mixtures containing DNA, it is perferable to keep the pH around 7 to 8.5 to retain biological activity. When deproteinizing mixtures containing RNA, it is preferable to keep the pH around 5.5 to 7 to retain biological activity. When deproteinizing mixtures containing high-molecular weight polymers such as nucleic acids or polysaccharides, it is preferable that the agitation be gentle and avoid processing which could generate shear forces.

Further description of the details of the invention is found in the following examples.

## EXAMPLE 1

### Adsorption and Removal of Proteins

To 10 ml of 0.1% bovine serum albumin protein (BSA) aqueous solution in a 15 ml polystyrene centrifuge tube, increasing amounts of azlactone-functional particles prepared according to Example 5D of European Patent Publication 0 392 735 (20 μm in diameter) were added and mixed for 10 minutes. The samples were filtered using a 0.2 μm disposable filter. The spectral absorbance of the filtrate using a Model 35 spectrophotometer available from Beckman Instruments of Palo Alto, CA, USA was measured at 280 nm where strong amino acid absorption bands are known, in order to determine the degree of deproteinization.

As is clear from the results shown in Figure 1, azlactone-functional particles bound and removed proteins from the proteinaceous, aqueous solution. The amount of proteins in the filtrate decreased with increasing amounts of azlactone-functional particles added. At 25 g of azlactone-functional particle/g of BSA, greater than 90% of the total mass of proteins was removed from the aqueous solution. The complete deproteinization of the aqueous solution could be accomplished by adding excess amounts of azlactone-functional particles or reducing the size of the azlactone-functional particles to increase surface area. From the slope of line 1 in Figure 1, the adsorption density was determined

as 0.038 g of BSA/g azlactone-functional particle (20 μm in diameter).

## EXAMPLES 2 AND 3

### Adsorption Kinetics of Proteins

To 10 ml of 0.1% bovine serum albumin (BSA) or 0.025% lysozyme in a 15 ml polystyrene centrifuge tube, two different amounts of azlactone-functional particles prepared according to the method referenced in Example 1 (20 g of particles/g BSA for Example 2 and 80 g of particles/g lysozyme for Example 3, respectively) were added and mixed for various periods of time. After incubation, the samples were filtered using a 0.2 μm disposable filter. The adsorbance of the filtrate was measured at 280 nm to determine the degree of deproteinization according to the procedure of Example 1.

As shown in Figure 2 for both Example 2 (line 2) and Example 3 (line 3), the adsorption of proteins on azlactone-functional particles was completed within a minute. This clearly suggests that the deproteinization process using azlactone-functional supports is efficient and effective. Figure 2 also shows that azlactone-functional particles can adsorb and remove both positively charged proteins (lysozyme) and negatively charged proteins (BSA). This ability is unexpected compared with conventional methods using ion-exchange media which can remove either net-positive proteins or net-negative proteins, but not both.

## EXAMPLE 4

### Deproteinization of Bacterial Lysate

A pure culture of a common soil bacterium, *Pseudomonas putida*, was grown in 500 mL of minimal salts medium (described in Stanier et al., *J. Gen. Microbial, Vol. 43, pp. 159-271 (1966))* containing succinic acid as the sole carbon/energy source. Exponentially-growing cells were harvested by centrifugation and were resuspended in 7 mL TE buffered aqueous solution (10 mM Tris, 1 mM EDTA, pH 8.0). The cells were lysed by four 15-second pulses of sonic oscillation using a Blackstone Model BP-2 Ultrasonic Probe available from Blackstone Ultrasonic Products, Inc. of Sheffield, PA with a power setting at 90. Unlysed cells and large particulate debris were removed by centrifugation at 15,000 x g for 20 minutes at 8°C. After overnight storage at -20°C, the cell lysate was thawed at room temperature, and then diluted to a final protein concentration of 1 mg/ml in PBS binding buffer aqueous solution (0.25 M phosphate buffer, pH 7.5; 0.15 M NaCl; 0.1% w/v Triton X-100 octoxynol commercially available from Rohm and Haas of Philadelphia, PA). The diluted cell lysate consisted of 3 parts crude cell lysate to 7 parts PBS binding buffer. Two ml of diluted cell lysate (1 mg

protein/ml) was added to 40 mg of azlactone-functional particles (prepared according to the method referenced in Example 1). The mixture was vortexed for 5 minutes and allowed to stand at room temperature for 1 hour. After the adsorption binding period, the particles were separated from the aqueous buffer by centrifugation in micro-centrifuge tubes.

A BCA protein assay commercially available from Pierce Chemical Co. of Rockford, IL was employed. The BCA protein assay showed an 80% reduction in BCA-reactive material in cell lysate (Table 1). Since this is a crude cell lysate, the BCA-reactive material that remained after azlactone-functional support separation treatment may be due to non-protein cell material. Thus, it is suggested that at least 80% of the protein is removed from a crude lysate under these conditions.

TABLE 1

Concentration of Proteins

| Sample | Concentration of Protein (mg/ml) |
|---|---|
| No protein control (after azlactone binding) | 0.00 |
| Diluted cell lysate (before azlactone binding) | 1.03 |
| Diluted cell lysate (after azlactone binding) | 0.21 |

An ultraviolet nucleic acid purity assay was employed using the following procedure developed by Warburg and Christian (Biochemische Zeitschrift, vol. 310, pg. 384 (1941)): small samples (10 µl were removed from the cell lysate/binding buffer solution before and after treatment with the azlactone-functional particles. The small samples were diluted 100-fold with deionized water and the resulting solutions were tested for their absorbance of ultraviolet light at 260 and 280 nm wavelengths using a Model 35 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA, USA).

This purity assay indicated that azlactone treatment led to a substantial increase in the purity of the nucleic acids in the cell lysate (Table 2). The 260/280 nm absorbance ratio of the diluted cell lysate (using the procedure according to Example 1) indicated only 15% nucleic acid purity before azlactone separation treatment. After azlactone separation treatment, the nucleic acid was nearly 100% pure (with respect to protein). This clearly demonstrates that azlactone-functional particles can be used to purify nucleic acids.

TABLE 2

Purity of Nucleic Acids.

| Sample | Absorbance 260/280 nm |
|---|---|
| Diluted cell lysate (before azlactone binding) | 1.54 |
| Diluted cell lysate (after azlactone binding) | 2.00 |

EXAMPLE 5

Deproteinization of Biological Fluids Containing Recombinant DNA

When performing recombinant DNA manipulations of biological extracts, it is often necessary to alter DNA molecules using one enzyme under a particular set of conditions (such as pH value and $MgCl_2$, NaCl, KCl concentrations), and then to additionally modify the DNA using a second enzyme requiring a different set of conditions. One of the chief difficulties in performing these series of reactions, is that the first enzyme possesses altered specificity under the second set of conditions.

A common example is that of the restriction enzyme EcoRI, which cuts the DNA sequence GAATTC when used in standard conditions such as 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, and 100 mM NaCl. However, under lower salt concentration or higher pH, EcoRI will alter its specificity and cut the sequence AATT. This is commonly referred to as EcoRI* (EcoRI star) activity, and results in undesired degradation of the DNA molecules. Hence, the desirability of destruction/removal of EcoRI activity before changing the reaction conditions for the addition of a second enzyme.

The most common method of removing enzyme activity is through treatment of the solution with phenol. Unfortunately, the phenol must then be removed through extraction with chloroform, and the DNA precipitated with ethanol (see Molecular Cloning, T. Maniatis, E. F. Fritsch and J. Sambrook, Cold Spring Harbor, 1982). The procedure is laborious, and produces hazardous chemical wastes with expensive disposal costs.

The deproteinization of the DNA/enzyme mixture using azlactone-functional supports is an attractive alternative to phenol treatment. A preferred method is the use of azlactone-functional particles (prepared according to the method of Process III and Examples 5A-5K of European Patent Publication 0 392 735), either by direct addition of the beads to the DNA/enzyme mixture and their removal by centrifugation, or by passing the DNA/enzyme mixture through a minicolumn containing azlactone network beads.

As an example of this procedure, 1.0 μg of phage lambda DNA was cut with restriction enzyme HindIII in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 50 mM NaCl in a volume of 30 μl by incubation at 37°C for 40 minutes. All reactions described were in 400 μl polypropylene tubes, distributed by Biorad, (Richmond, CA), catalog number 223-9503. The reaction mixture was then passed through a minicolumn (a sterile, plastic automatic pipeter tip containing 1 mg of azlactone-functional beads). The solution was kept in contact with the azlactone-functional beads for 10 seconds, and the solution was slowly passed through the column during an additional 5 seconds. 0.2 μg of plasmid pBR322 DNA was added to 15 μl of the phage lambda/HindIII solution and incubated for an additional 4 hours at 37°C, then analyzed by gel electrophoresis. The pBR322 DNA was not digested by any remaining HindIII activity in the reaction mix, indicating that the HindIII was completely removed/inactivated by the azlactone treatment.

The NaCl concentration of the remaining 15 μl of HindIII digested lambda DNA was raised from 50 mM to 100 mM, and the DNA was digested with EcoRI for 40 minutes at 37°C, and analysis by gel electrophoresis indicated complete digestion. This shows that the exposure of the DNA sample to the azlactone-functional beads had no deleterious effect on subsequent EcoRI digestion.

A sample of the HindIII EcoRI digested DNA was run through an azlactone-functional bead minicolumn (as previously described), and the DNA was ethanol precipitated, and ligated overnight with T4 DNA ligase. Gel electrophoresis showed complete ligation of the sample, indicating that the single stranded ends of the DNA remained intact after exposure to the azlactone-functional beads.

This example shows that azlactone-functional beads can be used to remove undesired enzyme activities from biological samples. It has worked equally well with less purified DNA, prepared from a "10 minute" DNA purification system, described in Biotechniques (Feb., 90): volume 8, p172-173. The DNA is apparently unaffected by its exposure to azlactone-functional beads prepared according to Process III described above, and can be digested with additional enzymes, or ligated efficiently by T4 DNA ligase.

## EXAMPLE 6

### Deproteinization of Biological Fluids Containing Plasmids

As additional evidence that DNA is unaffected by exposure to azlactone-functional support, plasmid pUC118 was cut with Pst I as previously described. The sample was exposed to azlactone-functional beads as described, and the 100% cut pUC118 was ethanol precipitated, and ligated with T4 DNA ligase. the Pst I site cuts pUC118 in the sequence coding for beta-galactosidase, and any alteration of the nucleotide bases in this sequence would result in a bacterial

transformant which is Lac minus. The frequency of Lac minus transformants after azlactone exposure was between 0.2 to 0.5%, similar to the 0.2 to 0.9% seen in the phenol treated controls. Similar results were obtained with HindIII digested pUC118. This further demonstrates that DNA sequences are unaltered by exposure to azlactone network beads.

The use of azlactone-functional supports need not be limited to the inactivation of restriction enzymes. It is applicable to any situation where it is desirable to eliminate enzyme activities from biological samples or culture extracts. When scaling up to larger applications, it is important to determine the amount and degree of azlactone functionality on the surfaces of the azlactone-functional supports, since the presence of other proteins in the sample may compete with the enzyme for binding the azlactone-functional supports.

## EXAMPLE 7

## Use of Azlactone-functional Membranes for Deproteinization of Solutions

Samples of hydrophilized polyethylene microporous membrane grafted with vinyldimethylazlactone (VDM), hydroxyethylmethacrylate (HEMA), or a combination of VDM and HEMA and prepared according to Example 13 of International Patent Application Serial No. US 93/_____ (Gagnon et al.) (Attorney Docket No. 45471PCT2A) and samples of ungrafted membrane prepared according to Example 3 of the same application. 0.6 cm diameter discs of the membrane were cut and placed in a microcentrifuge tube in stacks of of 1, 2, or 3 discs in each tube. 0.2 ml of 0.1% bovine serum albumin protein solution was placed in the tube above the membrane stack, followed by filtering this solution through the membrane via centrifugal force. The BSA concentration in the filtrate was measured via ultraviolet spectroscopy at 280 nm, and calculated the percentage removed. The results are tabulated in Table 3 below:

TABLE 3

| %VDM | % HEMA | Wt % Grafting Add-On | # of Discs | % BSA Removed |
|---|---|---|---|---|
| 0* | 0 | 0 | 1 | 8 |
| 0* | 0 | 0 | 2 | 9 |
| 0* | 0 | 0 | 3 | 7 |
| 0* | 10 | 83.5 | 1 | 17 |
| 0* | 10 | 83.5 | 2 | 15 |
| 0* | 10 | 83.5 | 3 | 6 |
| 0* | 25 | 167.4 | 1 | 19 |
| 0* | 25 | 167.4 | 2 | 17 |
| 0* | 25 | 167.4 | 3 | 9 |
| 50** | 0 | 139.3 | 1 | 52 |
| 50** | 0 | 139.3 | 2 | 68 |
| 50** | 0 | 139.3 | 3 | 92 |
| 10 | 25 | 105.4 | 1 | 26 |
| 10 | 25 | 105.4 | 2 | 87 |
| 10 | 25 | 105.4 | 3 | 100 |

* Comparison Examples

** The filtration rate for this VDM-only sample was quite slow due to the hydrophobicity of the VDM-functionalized surface.

As can be readily seen by comparing the VDM grafted examples with the Comparison Examples, the percent of BSA removed in the VDM-grafted membranes was several times greater than for the ungrafted membranes or the HEMA-only-grafted membranes. Further, the use of multiple discs of azlactone-functional membrane stacked in the microcentrifuge tube improved deproteinization. Use of a co-graft of VDM and HEMA achieved 100% deproteinization with three discs.

The invention is not limited to these embodiments. The following claims identify the scope of the invention.

What is claimed is:

1. A deproteinization method, comprising separating proteinaceous materials from non-proteinaceous materials by binding proteinaceous materials to an azlactone-functional support.

2. The method according to Claim 1, further comprising the steps of:

(a) contacting an azlactone-functional support with the biological fluid to bind proteinaceous materials to said azlactone-functional support;

(b) separating said azlactone-functional support having proteinaceous materials bound thereto from said fluid containing said non-proteinaceous materials.

3. A method for controlling restriction enzyme activities in biological fluid comprising non-proteinaceous, genetic material and restriction enzyme, comprising:

(a) contacting azlactone-functional support with said biological fluid;

(b) inactivating said restriction enzyme in said biological fluid by binding said restriction enzyme to said azlactone-functional support; and

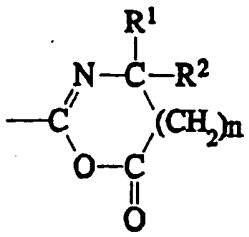
(c) separating said azlactone-functional support from said biological fluid to remove inactivated restriction enzyme from said biological fluid.

4. The method according to Claim 3, wherein the method further comprises the steps of:

(d) digesting the non-proteinaceous, genetic material with a second enzyme; and

(e) ligating the digested, non-proteinaceous, genetic material with a ligase.

5. The method according to Claim 1, Claim 2, or Claim 3, wherein said azlactone-functional support is a material having units of the formula:



wherein $R^1$ and $R^2$ independently can be an alkyl group having 1 to 14 carbon atoms, a cycloalkyl group having 3 to 14 carbon atoms, an aryl group having 5 to 12 ring atoms, an arenyl group having 6 to 26 carbon and 0 to 3 S, N, and nonperoxidic O heteroatoms, or $R^1$ or $R^2$ taken together with the carbon to which they are joined can form a carbocyclic ring containing 4 to 12 ring atoms, and n is an integer 0 or 1.

6. The method according to Claim 5, wherein said proteinaceous materials and non-proteinaceous materials are in a biological fluid.

7. The method according to Claim 6, wherein said azlactone-functional support are particles mixed into the biological fluid for binding of proteinaceous materials to said particles, and wherein said particles are removed from said biological fluid after said binding.

8. The method according to Claim 5, wherein said azlactone-functional support is an article which contacts said biological fluid for binding of proteinaceous materials to said article, and wherein said azlactone-functional article comprises a membrane or web.

9. The method according to Claim 1 or Claim 2, wherein said proteinaceous materials comprise whole blood, plasma, sera, lymph, bile, urine, fermentation liquor, spinal fluid, sputum, sweat, microbial culture filtrate, biological extracts, cell lysate, and combinations thereof.

10. The method according to Claim 1 or Claim 2, wherein said proteinaceous material binds to said azlactone-functional support at a pH value of between about 0 and about 11 and at a temperature from about 0°C to about 100°C within a reaction time of about 1 to about 10 minutes.

11. The method according to Claim 2, wherein said separating comprises centrifugation, skimming, filtration, or sedimentation.

12. The method according to Claim 3, wherein the non-proteinaceous, genetic material comprises nucleic acids.

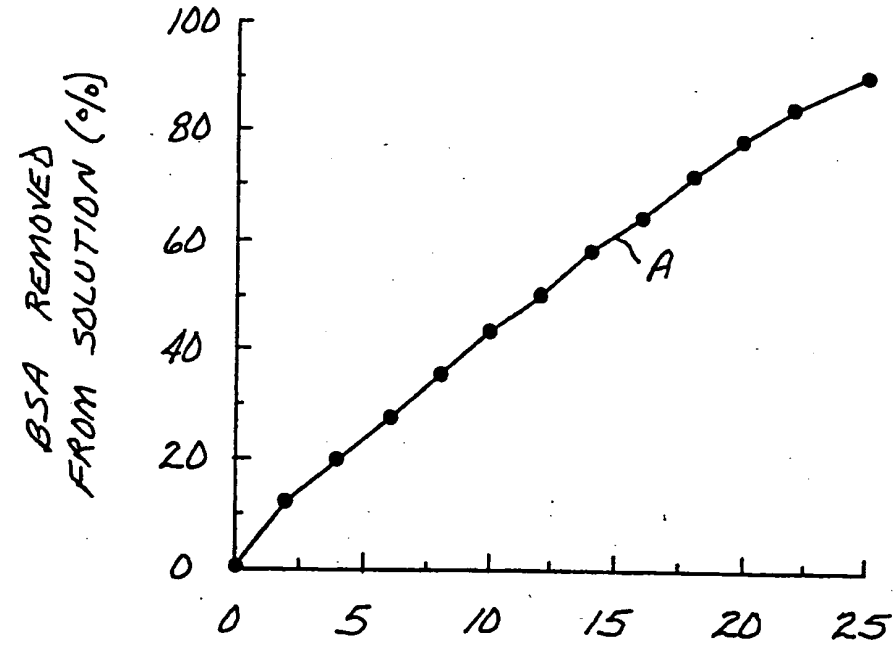

BSA REMOVED FROM SOLUTION (%)
100
80
60
40
20
0
A
0
5
10
15
20
25
AZLACTONE FUNCTIONAL PARTICLE (g/g BSA)

Fig. 1

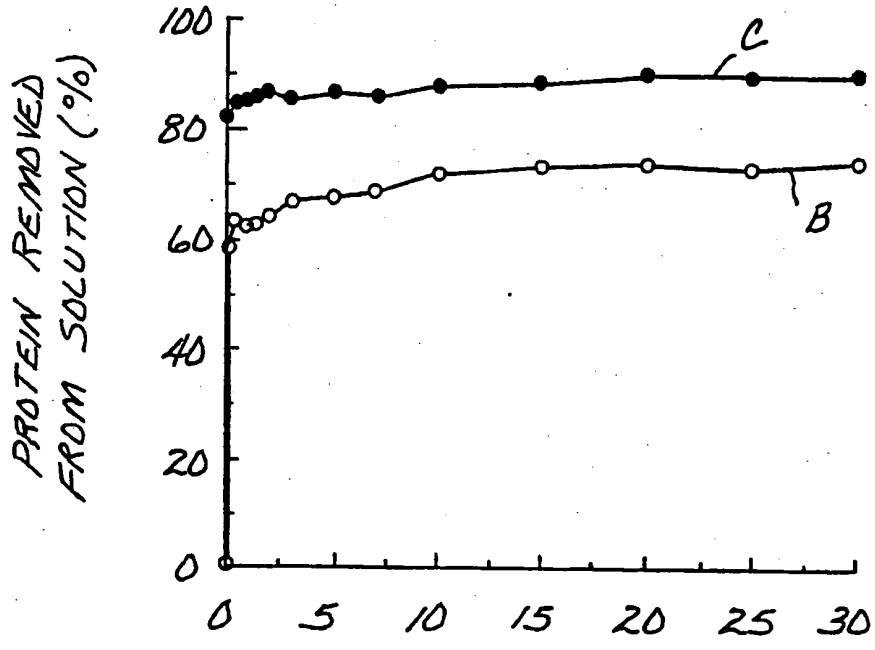

PROTEIN REMOVED FROM SOLUTION (%)
100
80
60
40
20
0
C
B
0
5
10
15
20
25
30
INCUBATION TIME (MIN.)

Fig. 2

International Application No PCT/US 93/04576

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07H1/08; C12N9/99; C12Q1/68; C07K3/24

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | C07H ; C12N ; C12Q ; C07K B01J |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category ° | Citation of Document,[11] with indication, where appropriate, of the relevant passages[12] | Relevant to Claim No.[13] |
|---|---|---|
| A | WO,A,9 010 637 (DU PONT DE NEMOURS) 20 September 1990 see the whole document & US,A,4 923 978 cited in the application | 1-2,5-12 |
| A | WO,A,9 207 879 (MINNESOTA MINING & MANUFACTURING CO.) 14 May 1992 see the whole document | 1-2,5-12 |
| A | EP,A,0 392 735 (MINNESOTA MINING AND MANUFACTURING CO.) 17 October 1990 cited in the application see the whole document | 1-2,5-12 |

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 26 AUGUST 1993 | 23 -09- 1993 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | P. Masturzo |

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category ° | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| A | EP,A,0 392 783 (MINNESOTA MINING AND MANUFACTURING CO.)<br>17 October 1990<br>cited in the application<br>see the whole document | 1-2,5-12 |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9304576
SA 74688

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 26/08/93

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-9010637 | 20-09-90 | US-A- | 4923978 | 08-05-90 |
| | | AU-B- | 625639 | 16-07-92 |
| | | AU-A- | 3352589 | 09-10-90 |
| | | AU-B- | 632284 | 24-12-92 |
| WO-A-9207879 | 14-05-92 | US-A- | 5200471 | 06-04-93 |
| | | CA-A- | 2093641 | 06-05-92 |
| | | EP-A- | 0556271 | 25-08-93 |
| EP-A-0392735 | 17-10-90 | JP-A- | 2294308 | 05-12-90 |
| EP-A-0392783 | 17-10-90 | US-A- | 5013795 | 07-05-91 |
| | | AU-B- | 617047 | 14-11-91 |
| | | AU-A- | 5294090 | 11-10-90 |
| | | CA-A- | 2014156 | 10-10-90 |
| | | JP-A- | 3054211 | 08-03-91 |